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Characterization and Classification of Strains of *Francisella tularensis* Isolated in the Central Asian Focus of the Soviet Union and in Japan

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The two subspecies of *Francisella tularensis*, *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *palaearctica* (type B), differ from each other in biochemistry and virulence. Strains of *F. tularensis* subsp. *tularensis* are believed to be confined to North America, whereas strains of *F. tularensis* subsp. *palaearctica* occur in Europe, in Asia, and in North America. Moreover, the existence of two other subspecies, designated *F. tularensis* subsp. *mediaasiatica* and *F. tularensis* subsp. *palaearcitica japonica*, has been suggested for strains of *F. tularensis* isolated in the central Asian focus of the Soviet Union and in Japan, respectively. In the present study, strains biochemically classified as *F. tularensis* subsp. *mediaasiatica* or *F. tularensis* subsp. *palaearctica japonica* have been investigated by hybridization with probes specific to 16S rRNAs of the two main subspecies. Furthermore, the virulence and biochemical characteristics of the strains were compared with those of strains belonging to *F. tularensis* subsp. *palaearctica* and *F. tularensis* subsp. *tularensis*. It was found that 16S rRNAs of *F. tularensis* subsp. *mediaasiatica* and *F. tularensis* subsp. *palaearctica japonica* hybridize with the probe specific to a genotype proposed herein, genotype A (*F. tularensis* subsp. *tularensis*), which shows that strains genetically related to this subspecies are found outside North America. However, the central Asian strains differed from *F. tularensis* subsp. *palaearctica* and *F. tularensis* subsp. *tularensis* strains when investigated by fermentation of glucose. The results of the biochemical tests could not be unambiguously used for differentiation of strains into *F. tularensis* subsp. *palaearctica* or *F. tularensis* subsp. *tularensis*. These drawbacks suggest that classification of strains of *Francisella* on the basis of 16S rRNA analysis may be preferable to classification on the basis of biochemical analysis.

The gram-negative bacterium *Francisella tularensis*, the cause of the zoonotic disease tularemia, is widely distributed in the Northern hemisphere. Numerous infections in humans have occurred over relatively large areas in the United States, in Europe, and in Asia. In 1959, the division of *F. tularensis* into *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *palaearctica* (type B) was suggested and has been officially used thereafter (2, 18). *F. tularensis* subsp. *tularensis* is believed to be confined to North America. It is highly virulent and causes severe illness in mammals (23). This subspecies is associated with tick-borne tularemia in rabbits (2, 11). *F. tularensis* subsp. *palaearctica* is found in Asia, in Europe, and also in North America. This subspecies is frequently linked to waterborne disease of rodents (2, 11). It is less virulent for mammals than *F. tularensis* subsp. *tularensis* (23).

In 1970, Soviet investigators proposed the alternative designations *F. tularensis* subsp. *nearctica* for *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* for *F. tularensis* subsp. *palaearctica* (2, 18, 19). Two other subspecies were also suggested: *F. tularensis* subsp. *mediaasiatica* for strains isolated in the central Asian focus of the Soviet Union and *F. tularensis* subsp. *palaearctica japonica* for strains isolated in Japan (11).

The identities of strains of *F. tularensis* can be verified by agglutination with specific antisera. However, since strains of *Francisella* seem to have similar antigenic compositions (15), it has not been possible to distinguish the different subspecies from each other by antigenic identification. Instead, subspecies of *Francisella* have been classified by biochemical tests (1, 12) and, recently, also by using probes specific to the 16S rRNAs of each of the two main subspecies (3). In the present study, strains isolated in central Asia and in Japan and designated hereafter as *F. tularensis* subsp. *mediaasiatica* and *F. tularensis* subsp. *palaearctica japonica*, respectively, were investigated by biochemical assays and by using probes specific for each of the two main subspecies.

MATERIALS AND METHODS

Bacteria. The *Francisella* strains used in this study are listed in Table 1. All strains were grown for 3 days on modified Thayer-Martin agar plates containing Gc medium base (36 g/liter; Difco Laboratories, Detroit, Mich.), hemoglobin (10 g/liter; Difco), and IsoVitaleX (100 mg/liter; BBL Microbiology Systems, Cockeysville, Md.) at 37°C with 5% CO_2 in air.

Estimation of citrulline ureidase activity. A 1.0-ml sample of a bacterial suspension (10^{10} bacteria) in 0.1 M phosphate-buffered saline (pH 6.5) was mixed with 1.0 ml of 0.7% (wt/vol) L-citrulline (Sigma Chemical Co., St. Louis, Mo.), and the mixture was incubated for 20 h at 30°C. An aliquot (0.01 ml) of the mixture was removed and mixed with 0.49 ml of distilled water, 1.0 ml of freshly prepared ninhydrin reagent (625 mg of ninhydrin [Sigma]) in 10 ml of 6 M H_3PO_4 and 15 ml of glacial acetic acid), and 1.5 ml of acetic acid. Samples were boiled for 1 h, cooled to room temperature, and adjusted to 7 ml with acetic acid. The conversion of

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TABLE 1. Bacterial strains used in this study

Subspecies and description	Source and/or description (reference)
F. tularensis subsp. *palaearctica* (*holarctica*[a])	U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md.; live vaccine strain
F. tularensis subsp. *palaearctica* (*holarctica*[a])	Isolated from a human with tularemia meningitis (6)
F. tularensis subsp. *tularensis* (*nearctica*[a])	S. J. Stewart, Rocky Mountain Laboratory, Hamilton, Montana; isolated from tick
F. tularensis subsp. *tularensis* (*nearctica*[a])	ATCC 6223; reference strain for *F. tularensis* subsp. *tularensis* (B38)
F. tularensis subsp. *mediaasiatica*; Russian strain 543	Isolated in the central Asian focus, USSR, in 1965; isolated from gerbil
F. tularensis subsp. *mediaasiatica*; Russian strain 120	Isolated in the central Asian focus, USSR, in 1965; isolated from hare
F. tularensis subsp. *mediaasiatica*; Russian strain 240	Isolated in the central Asian focus, USSR, in 1982; isolated from tick
F. tularensis subsp. *palaearctica japonica*	Isolated in Japan in 1926; isolated from a human lymph node

[a] Alternative names proposed by Soviet scientists in 1970 (2).

L-citrulline to ornithine was spectrophotometrically recorded at 490 nm and was judged positive when >15 μmol of ornithine was produced after 20 h.

Fermentation of glucose and glycerol. Production of acid was estimated in a liquid medium containing heart infusion broth (22.5 g/liter; Difco), cysteine-HCl (1.8 g/liter), $FeSO_4 \cdot 7H_2O$ (0.45 g/liter), L-histidine (0.9 g/liter), KCl (0.18 g/liter), thiamine-HCl (4.5 mg/liter), 0.02% (wt/vol) hemin, 0.12% (wt/vol) bromthymol blue, and 2.5% normal human serum. The pH of the medium was adjusted to 7.6.

RNA hybridization. The procedure for RNA hybridization was essentially as reported elsewhere (8). The probes and their utilization for identification of *Francisella* strains have been reported by Forsman et al. (3).

Virulence tests. To investigate virulence, groups of five mice each were intraperitoneally injected with 10^6 bacteria of each of the *F. tularensis* strains used in this study. This dose was lethal to challenged animals for all bacterial strains used, in the case of ATCC 6223. The virulence for rabbits was tested with one rabbit for each strain by subcutaneous injection of 10^6 bacteria. This dose of *F. tularensis* subsp. *tularensis* is known to be lethal to rabbits, whereas the same dose of *F. tularensis* subsp. *palaearctica* is not (12, 17–19).

RESULTS AND DISCUSSION

Strains classified as *F. tularensis* subsp. *mediaasiatica* (isolated in the central Asian part of the Soviet Union) and *F. tularensis* subsp. *palaearctica japonica* (isolated in Japan) were investigated for their relationships to strains of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *palaearctica*, respectively. The strains were compared with strain B38, which is the ATCC type strain for *F. tularensis* subsp. *tularensis*, and the live vaccine strain *F. tularensis* LVS, the best studied of the strains belonging to *F. tularensis* subsp. *palaearctica* (Table 1). These strains are, however, attenuated, either by long-term passage on artificial media or by active attenuation on medium containing serum antibodies (20). It is unknown whether these strains have retained their original phenotypes. Because of this uncertainty, two virulent strains showing their original properties, representing each of the two main subspecies of *F. tularensis*, were included. The *F. tularensis* subsp. *palaearctica* strain was isolated from a patient with meningitis in Sweden, and the *F. tularensis* subsp. *tularensis* strain was isolated from a tick found on a hare trapped in Canada (Table 1).

Francisella strains are known to display similar protein profiles (22), fatty acids (9, 14), antigenic composition (15), and behavior in biochemical tests (1, 12). Nevertheless, *F. tularensis* subsp. *tularensis* may be distinguished from *F. tularensis* subsp. *palaearctica* by its capacity to produce acid in medium containing glycerol as a carbon source (2), its possession of the enzyme citrulline ureidase (12), and its ability to hybridize to an oligonucleotide specific to 16S rRNA of the subspecies (3).

Bacteria of the *F. tularensis* strains were subjected to RNA colony hybridization. The hybridization was performed for each of the *Francisella* strains with two oligonucleotides specific to 16S rRNAs of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *palaearctica*, respectively (3). The 16S rRNAs of the strains belonging to *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *palaearctica* showed the expected patterns of hybridization. The *F. tularensis* subsp. *mediaasiatica* and *F. tularensis* subsp. *palaearctica japonica* strains hybridized to the probe specific to *F. tularensis* subsp. *tularensis* (Table 2). All *Francisella* strains used in this study hybridized to a third probe that is thought to be genus specific (3). No hybridization to *Escherichia coli* DH1 was found.

It has been suggested that the presence of the enzyme citrulline ureidase correlates with the virulence of strains of *F. tularensis* subsp. *tularensis* (12). However, this enzyme has also been demonstrated in the virulent strain of *F. tularensis* subsp. *palaearctica* included in this study (6). The attenuated strains of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *palaearctica* and the strain of *F. tularensis* subsp. *palaearctica japonica* lacked this enzyme activity. The virulent strains of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *palaearctica* as well as the three strains of *F. tularensis* subsp. *mediaasiatica* showed citrulline ureidase activity (Table 2).

A virulent strain is defined as a strain for which the median infective dose subcutaneously injected is 1 to 10 organisms for mice, guinea pigs, and rabbits. To distinguish *F. tularensis* subsp. *tularensis* from *F. tularensis* subsp. *palaearctica* in terms of virulence, the major disparity is the low virulence for rabbits of *F. tularensis* subsp. *palaearctica* (2, 11, 12). Accordingly, the virulent strain of *F. tularensis* subsp. *tularensis* showed a 50% lethal dose of <10 bacteria when injected subcutaneously in rabbits. The virulence of *F. tularensis* subsp. *mediaasiatica* and *F. tularensis* subsp. *palaearctica japonica* in rabbits was found to be $>10^6$.

As expected (2, 7), strains of *F. tularensis* subsp. *palaearctica* and strains of *F. tularensis* subsp. *tularensis* produced acid in medium containing glucose as a carbon source, whereas only strains of *F. tularensis* subsp. *tularensis* fermented glycerol (Table 2). The three *F. tularensis* subsp. *mediaasiatica* strains were found to produce acid from glycerol but not from glucose (Table 2), supporting earlier findings (2). In conclusion, *F. tularensis* subsp. *mediaasiatica* seems to be more closely related to *F. tularensis* subsp. *tularensis* than to *F. tularensis* subsp. *palaearctica*, because

TABLE 2. Biochemical and genetic characterization of *Francisella* strains

Subspecies (source or description)	Genotype (by hybridization)[a]	Citrulline ureidase activity	Glycerol fermentation	Glucose fermentation
F. tularensis subsp. *palaearctica* (*holarctica*[b])[c]		−	−	+
F. tularensis subsp. *tularensis* (*nearctica*[b])[c]		+	+	+
F. tularensis subsp. *palaearctica* (live vaccine strain)	B	−	−	+
F. tularensis subsp. *palaearctica* (isolated from human)	B	+	−	+
F. tularensis subsp. *tularensis* (ATCC 6223)	A	−	+	+
F. tularensis subsp. *tularensis* (isolated from tick)	A	+	+	+
F. tularensis subsp. *mediaasiatica* (isolated from gerbil)	A	+	+	−
F. tularensis subsp. *mediaasiatica* (isolated from hare)	A	+	+	−
F. tularensis subsp. *mediaasiatica* (isolated from tick)	A	+	+	−
F. tularensis subsp. *palaearctica japonica* (isolated from human lymph node)	A	−	+	+

[a] Two probes specific to 16S rRNAs of genotype A (*F. tularensis* subsp. *tularensis*) and of genotype B (*F. tularensis* subsp. *palaearctica*) were used.
[b] Alternative names proposed by Soviet scientists in 1970 (2).
[c] Biochemical characterization according to *Bergey's Manual of Systematic Bacteriology* (2).

it hybridizes to a probe specific to 16S rRNA of *F. tularensis* subsp. *tularensis*, possesses the enzyme citrulline ureidase, and produces acid in medium containing glycerol as a carbon source. However, the lack of fermentation of glucose distinguished the three strains of *F. tularensis* subsp. *mediaasiatica* from *F. tularensis* subsp. *tularensis*.

The causative agent of tularemia in Japan, *F. tularensis* subsp. *palaearctica japonica*, seems to be of low virulence (16), similar to other *F. tularensis* subsp. *palaearctica* strains (2, 12). Japanese strains also lack the enzyme citrulline ureidase but produce acid in medium containing either glucose or glycerol (19) (Table 2). These results were confirmed in the present study, but the Japanese strain (Table 2), as well as six other strains from Japan, hybridized to a probe specific to 16S rRNA of *F. tularensis* subsp. *tularensis*.

The presence of highly species-specific regions of 16S rRNA makes the use of oligonucleotides complementary to such regions of 16S rRNA a potent and reliable tool for discrimination of species and subspecies (3–5, 21). Previously, an *F. tularensis*-specific probe has been described (3). All strains used in the present study hybridized to this species-specific probe. In contrast to a previous suggestion of subspecies classification (2), the analysis of 16S rRNA showed that *F. tularensis* strains isolated in the central Asian focus of the Soviet Union and in Japan had a genotypic relationship to *F. tularensis* subsp. *tularensis*. Although biochemical tests can be used to discriminate among subspecies of *F. tularensis* strains (1, 2, 12), such tests have obvious drawbacks for unambiguous classification. For instance, it is unknown to what extent long-term passage on artificial media affects the phenotypes of *F. tularensis* strains. The type strain B38 (ATCC 6223) of *F. tularensis* subsp. *tularensis* does not exhibit the enzyme citrulline ureidase, in contrast to highly virulent strains of this subspecies (12). When isolated, strain B38 was highly virulent but became avirulent after several passages on artificial media (10). It may be presumed that this strain originally possessed citrulline ureidase activity. Furthermore, citrulline ureidase activity has been found in strains belonging to *F. tularensis* subsp. *tularensis* (12), *F. tularensis* subsp. *palaearctica* (6), and *F. tularensis* subsp. *mediaasiatica* (2), and its validity as a test to classify *F. tularensis* strains could thus be questioned.

In conclusion, the present study shows that the differentiation of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *palaearctica* by biochemical tests is by no means unambiguous. Furthermore, the aim of this study, to classify and characterize *F. tularensis* strains isolated in the central Asian focus of the Soviet Union and in Japan, added further complexity to the subspecies differentiation of *F. tularensis* strains when the results of the biochemical tests were considered. It appears that the biochemical characteristics and the virulence of *F. tularensis* strains do not reflect their genetic relationships, as judged by 16S rRNA. It may be useful, in order to avoid future ambiguity, to assess the relationships of *Francisella* strains by means of their genetic resemblances. On the basis of this criterion, the strains included in this study that were isolated in the central Asian focus of the Soviet Union or in Japan belong to a proposed genotype, genotype A (*F. tularensis* subsp. *tularensis*), irrespective of the fact that their virulence and some of their biochemical characteristics conform to those of genotype B (*F. tularensis* subsp. *palaearctica*) strains.

By determination of genotype, strains isolated outside North America will also be classified as genotype A. This is in contrast to the present view that strains with properties of *F. tularensis* subsp. *tularensis* are not found outside North America. However, the present results do not exclude the general view that highly virulent strains of *F. tularensis* are restricted to North America. To determine whether the proposed division of strains of *F. tularensis* into genotype A or B is advantageous and unambiguous, a more extensive study of *F. tularensis* strains has to be carried out. Moreover, it would be of interest to determine whether this classification can be extended to other members of the genus *Francisella*.

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